

Saturation recovery EPR and ELDOR at W-band for spin labels

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A reference arm W-band (94 GHz) microwave bridge with two sample irradiation arms for saturation recovery (SR) EPR and ELDOR experiments is described. Frequencies in each arm are derived from 2 GHz synthesizers that have a common time-base and are translated to 94 GHz in steps of 33 and 59 GHz. Intended applications are to nitroxide radical spin labels and spin probes in the liquid phase. An enabling technology is the use of a W-band loop-gap resonator [1]. The high efficiency parameter ($22 \text{ GW}^{-1/2}$) permits the saturating pump pulse level to be just 5 mW or less. Multifrequency measurements of T_1 previously reported in the range of 2 to 35 GHz [2] have been extended to 94 GHz. In all samples, the value of T_1 decreases at 94 GHz relative to values at 35 GHz, indicating the onset of a relaxation mechanism previously unreported in the liquid phase. Applications of SR EPR and ELDOR to the hydrophilic spin label 3 carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy (CTPO) are described in detail. Other samples were 2,2,6,6-tetramethyl-4-piperidone-1-oxyl (TEMPONE), 5-, 12-, and 16-doxylstearic acid spin labels (5-, 12-, and 16-SASL), and cholestane spin label (CSL). In the SR ELDOR experiment, nitrogen nuclear relaxation transfers saturation from pumped to observed hyperfine transitions. SR ELDOR was found to be an essential method for measurements of the nitrogen nuclear relaxation time, T_{1n} , for small molecules such as TEMPONE. Free induction decay (FID) signals for small nitroxides at W-band are also reported.

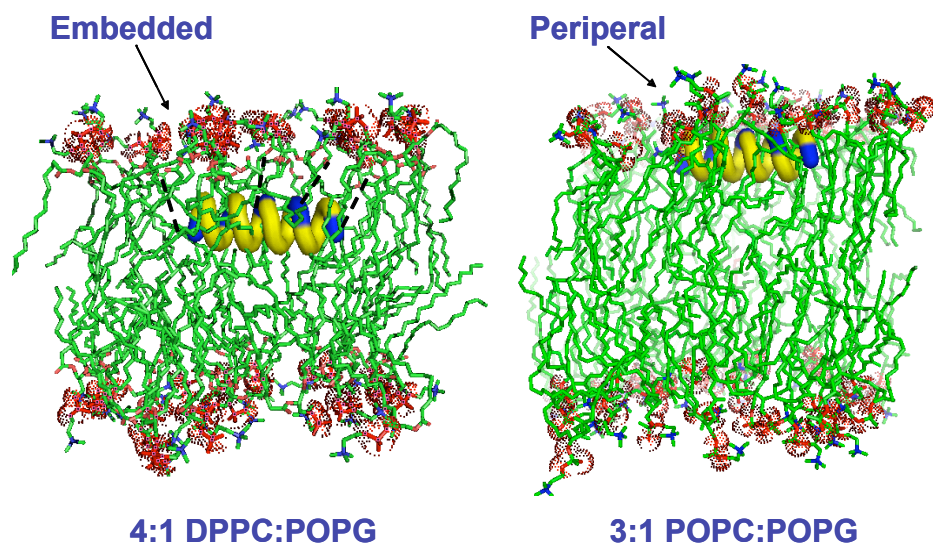
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Solid State NMR Studies of Protein Structure and Dynamics in Heterogeneous Environments

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We are developing and applying solid state NMR (ssNMR) techniques to the study of peptide structure and dynamics in unoriented membranes, immobilized on surfaces, and at varying levels of hydration. Many of these experiments rely on double-quantum filtering to simplify complex spectra and allow observation of specific interactions in peptides which comprise a small percentage of the sample under study. The advantages, challenges, and accuracy of these methodologies will be discussed along with experiments validating structural data obtained using model systems. Using these techniques we are studying peptide mimetics developed for lung surfactant replacement therapies. Lipid-associated proteins play key roles in determining the unique physical properties of pulmonary surfactant, yet little is known about their atomic-level structure and dynamics in the membrane environment. We are interested in the role specific protein sequences play in determining protein structure, membrane association, and ultimately the properties of the membrane. Using ssNMR we have characterized the structure of a peptide mimetic, KL₄, of lung surfactant protein B, SP-B, which is critical in forming a stable air-water interface in alveoli. With dipolar recoupling experiments, a preliminary structure of KL₄ interacting with POPC/POPG vesicles has been determined. Additionally, ³¹P and ²H NMR of the lipids indicate peptide-induced changes in the lipid dynamics. These studies point to the importance of high-resolution, atomic level characterization to understanding lipid-associated peptides and proteins in their native environments.



**Can a Protein Bound Zn(II)-OH₂ be Considered a Weak Acid?
The Answer will be Illustrated From of our Work on Zinc Metalloproteins**

Paul D Ellis, Andrew S. Lipton, and Robert Heck

After a brief introduction of our experimental approach, I would like to discuss the validity of the one of the paradigms of metallobiochemistry; namely can tetrahedral Zn²⁺ with water as ligand within a metalloprotein be considered as a weak acid? The question is rarely phrased in this fashion ... more often the question is asked what is the pK_a of the bound water. These questions arise from the usual experimental trick associated with mechanistic enzymology; namely determine the pH dependence of k_{cat}/K_M or simply k_{cat}. For zinc metalloproteins these curves are often bell shaped and one of the ionizations is often ascribed to the ionization of water bound to Zn²⁺. This idea has become a “scared cow” and as every knows the scared cows make the best hamburgers!

I will illustrate our thoughts on this issue with examples from our work on two zinc metalloproteins, LpxC and alkaline phosphatase.

Manganese and Iron Lipoxygenases: EPR Lineshape Analyses and Comparison

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The mononuclear metal center in lipoxygenases is the site of proton coupled electron transfer from a hydrocarbon substrate to metal, followed by channeled diffusion of oxygen to the radical substrate. Both iron and manganese forms of the enzyme are known. Manganese lipoxygenase forms an unusual product (a bis-allylic hydroperoxide), raising the question of whether the metal centers in the two classes of lipoxygenase are analogous. A hallmark of ferric lipoxygenase is that flexibility in the iron coordination environment is reflected in the EPR spectra (a). Distributions about two basic sets of zero field splitting parameters describe the ferric spectra. The value of D for manganous lipoxygenase was assigned and is of reasonable magnitude when compared with other Mn and Fe redox active mononuclear centers in enzymes (b). Recent examination of temperature dependence of manganous lipoxygenase W-band EPR spectra will be presented and the effects of distributions in D and E on these spectra will be compared with the situation in ferric lipoxygenase.

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***Mycobacterium tuberculosis* WhiB3 responds to dormancy signals through its [4Fe- 4S] cluster.**

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A fundamental challenge in the redox biology of *Mycobacterium tuberculosis* (Mtb) is to understand the mechanisms involved in sensing redox signals such as oxygen (O₂), nitric oxide (NO) and nutrient depletion, which are thought to play a crucial role in persistence. Here we show that *Mtb* WhiB3 responds to the dormancy signals NO and O₂ through its iron-sulfur (Fe-S) cluster. In order to functionally assemble the WhiB3 Fe-S cluster, we identified and characterized the *Mtb* cysteine desulfurase and developed a dedicated enzymatic reconstitution system for restoring Fe-S clusters in *Mtb*. Electron Paramagnetic Resonance (EPR) and UV-visible (UV-Vis) spectroscopy analysis of reduced WhiB3 is consistent with a one electron reduction of EPR silent [4Fe-4S]²⁺ to EPR active [4Fe-4S]¹⁺. Exposure of reconstituted WhiB3 to atmospheric O₂ caused gradual degradation of the [4Fe-4S]²⁺ cluster to generate a [3Fe-4S]¹⁺ intermediate. Importantly, this mechanism of cluster degradation is similar to that of the *E. coli* Fumarate Nitrate regulator (FNR). Furthermore, EPR analysis demonstrates that NO forms protein-bound dinitrosyl-iron-dithiol complex (DNIC) with the Fe-S cluster, indicating that NO specifically targets the WhiB3 Fe-S cluster. Our results establish for the first time a role for WhiB3 in sensing the physiologically relevant host signaling molecules NO and O₂ through its [4Fe-4S] cluster. These findings have major implications for the mechanism of redox sensing in *Mtb*.

MULTIFREQUENCY EPR STUDIES ON THE MN(II) CENTERS OF OXALATE DECARBOXYLASE

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Oxalate decarboxylase from *Bacillus subtilis* is composed of two cupin domains each of which contains a Mn(II) ion coordinated by four identical conserved residues. The similarity between the two Mn(II) sites has precluded previous attempts to distinguish them spectroscopically and complicated efforts to understand the catalytic mechanism [1]. A multifrequency cw-EPR approach has shown that two major spectroscopically distinct Mn(II) species are present in equal proportions in the resting state of the enzyme in HMTA storage buffer [2]. The main difference between these two species is the value of the fine structure parameters with $D_I = 1200$ MHz, $D_{II} = 2700$ MHz, and $E/D = 0.21$ for both sets. When the enzyme is placed in acetate buffer pH 5.2 or when formate is added, D_{II} is reduced to 2150 MHz and $E_{II}/D_{II} = 0.05$ while D_I and E_I remain the same indicating that only one Mn(II) is solvent accessible. Based on published crystal structure data, we conclude site I is the C-terminal Mn site while site II is the solvent-exposed N-terminal site and, therefore, the site of small molecule (acetate and formate) binding in agreement with recently published crystal structure data [3,4]. Multifrequency EPR experiments were performed on oxalate decarboxylase buffered over a pH range from 4.0 to 8.5 and showed subtle changes in the fine structure of both sites.

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Coordination Chemistry at the Fe(II) Site of Taurine/ α -Ketoglutarate Dependent Hydroxylases

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One- and two-dimensional Electron Spin Echo Envelope Modulation (ESEEM) experiments have been used to study the coordination chemistry at the Fe(II) site of taurine/ α -ketoglutarate (α KG) dioxygenase (TauD), a non-heme Fe(II) Hydroxylase. To facilitate EPR experiments, Fe(II)-NO derivatives of the enzymes were studied. The NO serves as a surrogate for molecular oxygen and spin-couples to the integer spin Fe(II) to yield an $S = 3/2$ paramagnetic center with a nearly axial EPR spectrum characterized by $g_{\perp} \approx 4.00$ and $g_{\parallel} = 2.00$. One-dimensional ESEEM spectra taken across the EPR lineshape showed modulations from ^{14}N and ^1H . At $g = 4$, the contributions from these coupled nuclei were overlapped making it necessary to use the two dimensional, 4-pulse HYSCORE method to resolve contributions from bound histidine nitrogens, coordinated H_2O , and ambient H_2O . HYSCORE spectra collected for samples in aqueous buffer and 60% $^2\text{H}_2\text{O}$ -buffer showed changes in H_2O and histidyl coordination as co-substrates, α KG and taurine, were added to the enzyme. Prior to co-substrate addition, HYSCORE spectra showed a substantial distribution of exchangeable, ^1H hyperfine couplings. When co-substrate α KG is added, the ^1H HYSCORE is considerably altered with better-defined cross-peaks arising from exchangeable, strong-coupled protons. Subsequent addition of substrate taurine to yield the ternary, Fe(II)-NO/ α KG/taurine, complex at the active site showed a new, ^1H hyperfine interaction that was not exchangeable in $^2\text{H}_2\text{O}$. The HYSCORE cross-peaks from this ^1H show a hyperfine tensor of axial symmetry characterized by a dipole-dipole distance of 3.2\AA and an isotropic contribution of 0.75 ± 0.3 MHz. Comparison of these data with the X-ray crystal structure of TauD and the results of parallel studies of TauD variants may provide important information on how active site protein residues work in concert with the Fe(II) center to catalyze the specific hydroxylation of taurine.

EPR/DEER Studies of the Structure of Erythrocyte CDB3 Hereditary Spherocytosis Variant P327R: Band 3 Tuscaloosa

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Previous studies have shown that a single P327R point mutation in the cytoplasmic domain of band 3 (cdb3) protein, known as band 3 Tuscaloosa, leads to a reduction in protein 4.2 content of the erythrocyte membrane and hemolytic anemia. Recent low-resolution studies have shown that this point mutation does not dissociate the cdb3 dimer nor does it lead to large scale rearrangement of the protein structure. In order to better define the structural changes in cdb3 that lead to the hemolytic anemia phenotype, site-directed spin labeling (SDSL), in combination with continuous wave electron paramagnetic resonance (EPR) and pulsed double electron-electron resonance (DEER) spectroscopies, has been employed to compare the structure of the R327 variant with wild type P327 cdb3. It is confirmed that the P327R mutation does not dissociate the cdb3 dimer nor does it change the spatial orientation of the two peripheral domains relative to the dimer interface. However, it does affect the packing of the C-terminal end of helix 10 of the dimerization arms in a subpopulation of cdb3 dimers, it leads to spectral changes at some residues in β -strand 11 and in the N-terminal end of helix10, and it produces measurable spectral changes at other residues that are near in space to the mutation site. The data indicate that the structural changes are subtle and are localized to one surface of the cdb3 dimer. The spectroscopic description of structural features of the P327R variant provides important clues about the location of one potential protein 4.2 binding surface on cdb3 as well as new insight into the structural basis of the membrane destabilization.

Pheromone Perception: Structure and Function of Pheromone Binding Protein

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The ability to respond to chemical stimuli is a fundamental behavior of all organisms. Lepidoptera male moths have an exquisitely sensitive olfactory system that is capable of perceiving airborne pheromone molecules released by females and responding to them over great distances. Olfactory signal transduction and information processing in insects (moths) are prime examples of chemical communication found in nature for its exquisite sensitivity and selectivity. Although not completely understood, extensive research on the biology and chemistry of this complex event has revealed many facets of olfaction where donors, recipients, enormous pool of chemicals/stimulators, binding/carrier proteins, and cellular receptors play their respective role with high precision, selectivity and sensitivity. Pheromone-binding proteins (PBPs), present in the antenna of male moth and other insect species, bind the volatile hydrophobic pheromone molecules and transport them across the aqueous sensillar lymph to the membrane-bound G protein-coupled receptor proteins.

The pH-dependent conformational change of the ApolPBP, the pheromone binding protein of silk moth *Antheraea polyphemus*, and its biological significance will be discussed (1-2). NMR investigation to address the issue of pheromone recognition, specificity and mechanisms of odor binding in ApolPBP is in progress in our laboratory. Our investigation of the hydrophobic pocket of ApolPBP with the hydrophobic probe ANS (amino naphthalene sulfonate) and AMA using 2-dimensional ^1H , ^{15}N -HSQC experiment will also be presented.

This research was supported by USDA grant 2003-35302-12930 (The Presidential Early Career Award for Scientists and Engineers to S.M.), and NSF grant IOS-0414073 (S.M.).

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Unraveling the Causative Role of Inducible Nitric Oxide Synthase- mediated Free Radical Production in the Pathogenesis of Diabetes

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Free radical production is implicated in the pathogenesis of *diabetes mellitus*. However, the mechanism by which oxidative stress is triggered and the identity of the oxidants involved is unknown. In this study, we used electron paramagnetic resonance (EPR) spectroscopy combined with *in vivo* spin trapping techniques to investigate the sources and mechanisms of free radical formation in rats. Free radical production was detected in the diabetic bile which correlated with lipid peroxidation and tissue damage in the liver and kidney. EPR spectra showed the trapping of a lipid-derived radical. Such radicals were demonstrated to be induced by hydroxyl radical through isotope labeling experiments. Multiple enzymes and metabolic pathways were examined as the potential source of the hydroxyl radicals using specific inhibitors. Xanthine oxidase, cytochrome P450s, *in vivo* Fenton reactions, or macrophage activation were not required for the production radical adducts. Interestingly, uncoupled inducible nitric oxide synthase was identified as the major source for radical generation. The specific iNOS inhibitor 1400W as well as L-arginine pretreatment reduced the EPR signals to baseline levels, unraveling the mechanism of hydroxyl radical production. Applying immunological techniques, we localized iNOS overexpression in the liver and kidney of diabetic animals. Taken together, our studies support inducible nitric oxide synthase as the primary source of reactive intermediates, which leads to lipid peroxidation and contributes to disease progression.

Using DEER to Investigate Conformational Motion of Transporters

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My laboratory uses spin labeling and EPR spectroscopy to map the motions underlying energy transduction and substrate translocation in three classes of transporters: two are multidrug efflux pumps and one is a sodium-dependent leucine transporter with sequence and functional similarity to human neurotransmitter transporters. DEER analysis of distance changes in the transport cycle reveals a rich and fascinating spectrum of conformational rearrangements. In the transporter MsbA, an ABC transporter putatively responsible for trafficking of lipid A across the bacterial inner membrane, large scale movements in three domains reflect a change in the orientation of a large substrate-binding chamber. The scale of the movement challenges prevailing mechanistic models of transport. In EmrE, a proton-coupled multidrug transporter, the conformational changes are of smaller amplitude. Analysis of distance distributions between pairs of spin labels suggests that substrate binding reduces the flexibility of the transporter possibly locking it into a unique conformation. I will discuss the caveats of the structural interpretation, the experimental difficulties of using DEER in membrane proteins and the use of distance constraints to determine high-resolution static structures.

Probing the Structural and Dynamic Properties of Integral Membrane Proteins with Solid-State NMR Spectroscopy and EPR Spectroscopy

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The Lorigan lab is using state-of-the-art magnetic resonance spectroscopic techniques to study the structural and dynamic properties of integral membrane proteins. Solid-state Nuclear Magnetic Resonance (NMR) and Electron Paramagnetic Resonance (EPR) spectra provide unique information on the structure, structural topology, dynamics, and membrane depth of several different membrane proteins. New alignment methods such as magnetically aligned phospholipid bilayers (bicelles) and phospholipid bilayer nanotube arrays are being developed for both solid-state NMR and EPR spectroscopic studies. NMR and EPR spectra from several different membrane proteins will be presented.

Cofactors of Electron Transfer Chain in Photosystem I – EPR Studies at Conventional and High Magnetic Fields

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Photochemical electron transfer reactions in Photosystems I and II take place within a series of cofactors that are associated with the reaction centers. In PSI, the two principal subunits PsaA and PsaB share similarities in their amino acid sequences and form a pseudo-symmetric structure that likely evolved from a homodimeric assembly. The electron transfer cofactors in PS1 include the primary electron donor (P700⁺), the primary electron acceptor A₀, and the phylloquinone A₁. We focused on two mutants, namely PsaA-Y696F and PsaB-Y676F, which break H-bonds to the A₀ chlorophyll. Changes in the environment due to these mutations are expected to be revealed by high-field EPR because of the improved g-tensor resolution at high magnetic fields. Though the high-field EPR data on A₀⁻ have left us inconclusive about mutational effects, a promising aspect of our results is that we can resolve the g-tensor components of the chlorophyll anion A₀⁻ and the phylloquinone anion A₁⁻ very clearly at high magnetic fields, whereas at X-band fields convolution of the A₁⁻ and A₀⁻ spectra is highly complicated and often leads to confusion. To distinguish between the radicals P700⁺ and the A₀⁻ anion at X-band, we have studied the power saturation effects to clearly identify the physical nature of these radical species. We present these results in detail comparing the advantage and disadvantages of the low and high magnetic fields. The EPR experiments at high field were performed at MW frequencies up to ~412 GHz using the facilities at the NHMFL in Tallahassee, FL.

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Topology and Helical Tilt Angle of Membrane Protein Determined in an Aligned Lipid Bilayer Media Using EPR Spectroscopy

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Magnetically aligned (bicelles) and mechanically aligned (glass-plate) phospholipid bilayers have been successfully used in a range of solid state NMR (SSNMR) and solution NMR studies to macroscopically order both membrane bound and water soluble macromolecules. Sample orientation enables the efficient high-resolution measurement of anisotropic spectral parameters that provide valuable structural and dynamic information for both EPR and NMR spectroscopic studies. In particular, several researchers have investigated membrane proteins and peptides incorporated into mechanically aligned phospholipids bilayers with solid-state NMR spectroscopy. However, for the first time we demonstrates the feasibility of obtaining topology and helical tilt information of an integral membrane protein inserted into various alignment media such as bicelles, nanomembranes and glass plates using a spin label EPR approach. A rigid nitroxide spin label attached to a helix of a protein was used to elucidate the structural topology and the tilt of the helix with respect to the membrane normal through the measurement of orientational dependent hyperfine splitting values. The advantages of using EPR to study the topology and helical tilt of membrane proteins in comparison to solid state NMR technique will be discussed.

Gd(III)-Nitroxide Pairs for Membrane Proteins Studies: A Multifrequency EPR Approach

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Distance measurements using site-directed spin labeling (SDSL) and EPR are based on magnetic interactions of a nitroxide spin-label with another paramagnetic center. The second center could be another nitroxide label or a paramagnetic metal ion. Previously, distance-dependent relaxation effects of Cu²⁺ (1) and Gd³⁺ (2) on nitroxides were measured with X-band (9 GHz) EPR and analyzed using Leigh's treatment (3). We are interested in extending the well-established method of SDSL EPR to high magnetic field experiments in order to fully utilize advantages of HF EPR. Here we report on experiments to investigate the interactions between Gd³⁺ ion positioned at the surface of the phospholipid bilayer and a nitroxide label attached to the transmembrane WALP peptide with X-band and W-band (95 GHz) EPR. Slow (as compared with other paramagnetic metal ions) electronic relaxation of Gd³⁺ at magnetic fields above 3 T and highest possible for an ion electronic spin state ($S=7/2$) results in easily observable relaxation enhancement effects for the nitroxide labels. We demonstrate that it is possible to manipulate the nitroxide-Gd³⁺ interactions by changing the magnetic field of the experiment: the electronic relaxation of Gd³⁺ slows with the field increase. We will also show that for nitroxide-labeled phospholipid bilayer the relaxation enhancement is anisotropic. The later measurements are attainable to the excellent angular resolution of HF EPR. T.I.S. acknowledges support from the NSF grant MCB-0451510. B.D was supported by Undergraduate Research Fellowship from NCSU and NSF REU Fellowship.

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Study of the Critical Structural and Catalytic Role of Arginine 160 in EutB Protein from Coenzyme B12-Dependent Ethanolamine Ammonia-Lyase

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The protein chemical, enzyme kinetic and electron paramagnetic resonance (EPR) spectroscopic properties of ethanolamine ammonia-lyase (EAL) from *Salmonella typhimurium* with site directed mutations in a conserved arginine residue at position 160 (R160) of the active site-containing EutB protein subunit have been characterized. R160 was predicted by our comparative model of EutB to play critical roles in protein structure and radical-mediated catalysis [Sun, L. and Warncke, K. (2006) *PROTEINS: Structure, Function, and Bioinformatics*, 64, 308-319]. The following mutant EutB proteins were prepared and evaluated: R160A, R160E, R160I, R160K. R160I and R160E mutants fail to assemble into an EAL oligomer that can be isolated by the standard enzyme purification procedure. The R160A and R160K EAL assemble, but R160A EAL is unstable and catalytically inactive. R160K EAL is active, but shows a 200-fold reduction in k_{cat}/K_M relative to wild type (WT). A comprehensive EPR and electron spin echo envelope modulation (ESEEM) spectroscopic protocol, in combination with ^2H - and ^{13}C -isotope labelling, is used to evaluate the degree of localization of mutation-induced changes in the structure of the reactants and protein in the active site region. R160K EAL forms Co^{II} -radical pair intermediates. The Co^{II} -radical pair separation distances are increased by 2 Å in R160K relative to WT, but the WT geometry among the deoxyadenosine C5'-methyl group and product radical C2 and C1 centers is conserved. The results show that residue R160 is critical in structuring the active site, and that the side chain guanidinium moiety plays a pervasive role in EAL catalysis, and in particular, in determining the relatively low barrier for radical rearrangement. The results provide strong support for the comparative model of EutB. Supported by NIH grant DK54514.

Accessing Interfacial Electrostatics of the Monolayer-Protected Gold Clusters by EPR: A Molecular Probe Approach

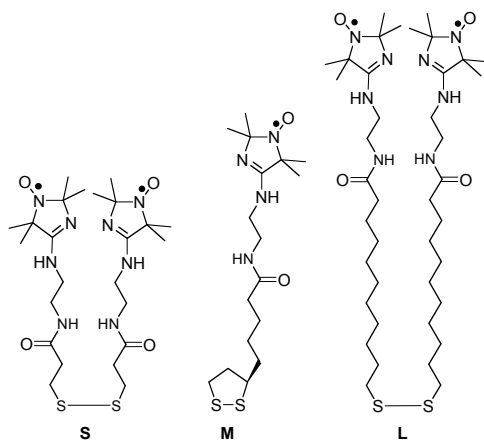
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Properties of monolayer-protected gold nanocluster's (MPCs) interface are generally manipulated through rational design and synthesis of the coating ligands. This is the key strategy for designing MPCs for biodiagnostics, bio- and chemical sensors, drug/DNA delivery, imaging, and biomolecular recognition purposes. Both stability and interactions of gold nanoparticles with each other and such biomolecules as proteins and DNA are governed by the surface potential resulting from the charge layer developed at the nanoparticle aqueous interface. To summarize, sensing, binding, and recognition capabilities of MPCs could be custom-tuned by properly controlling interfacial hydrophobic and electrostatic interactions. Local polarity could be probed by different analytical techniques including steady-state and time-resolved fluorescence, high-resolution NMR and EPR spectroscopy of nitroxides. Among those methods the molecular probe techniques offer the advantage of providing spectral readout directly from the position of the probe. Here, we describe newly synthesized pH-sensitive spin-labeled ligands and report on their use for probing electrostatics of the Au nanoparticle monolayer/water interface by EPR.



Halophilic Adaptation through High Salt NMR Studies

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Proteins from halophiles have adapted to challenging environmental conditions and require salt for their structure and function. How halophilic proteins adapted to a hypersaline environment is still an intriguing question. It is important to mimic the physiological conditions of the archae extreme halophiles when characterizing their enzymes, including structural characterization. The NMR derived structure of *Haloferax volcanii* dihydrofolate reductase in 3.5 M NaCl is presented and represents the first high salt structure calculated using NMR data. Structure calculations show that this protein has a solution structure which is similar to the previously determined crystal structure with a difference at the N-terminus of $\beta 3$ and the type of β -turn connection $\beta 7$ and $\beta 8$. Activity studies show that this protein is most active at 3.5 M NaCl and losses activity linearly down to 1.0M NaCl. This trend in activity has been investigated; however, there has been no good explanation for the protein's loss in activity. Relaxation data suggest that there is a reduction in the flexibility of this protein from 3.5 M NaCl to 1.0M NaCl. These data also suggest that there may be a change in the domain to domain motion which would effect the protein's activity.

Applications of High Accuracy Quantum Chemical Calculations of Magnetic Resonance Spectroscopic Properties

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Magnetic resonance spectroscopies (MRS) such as NMR, ESR and Mössbauer are very useful and powerful techniques to investigate biomolecular and model systems. In order to make quantitative use of these MRS properties, we have recently established a number of quantum chemical methods that can excellently predict numerous NMR, ESR and Mössbauer properties with theory-versus-experiment correlation $R^2 \sim 0.98$, covering a chemically diverse series of biomolecules, models, and drug molecules. Because of the high accuracy and broad coverage of these calculations, the results have enabled the correction of errors in experimental spectra, the assistance for full spectra assignment, and more importantly the refinement of metalloprotein x-ray structures. I will present a few recent applications to metalloproteins and metal complexes: 1) using the calculations of ^{13}C NMR hyperfine shifts to correct errors in the NMR spectra of deoxymyoglobin; 2) using the calculations of NMR hyperfine shifts to help fully assign the first solid-state NMR spectra of a high-spin ferrous complex; 3) using the calculations of ^{31}P NMR shifts to refine the geometry of a drug-binding site in farnesyl pyrophosphate synthase, determine the protonation state of an diphosphate substrate (which challenged the current textbook view) and the protonation state of the drug side-chain (which helped the discovery of new drug leads); 4) using the calculations of ESR and Mössbauer properties to refine the active site in nitrosyl hemoglobin (a paramagnetic system). These results open up the way for the quantitative analysis of NMR, ESR and Mössbauer spectra and the accurate structure determination of both diamagnetic and paramagnetic proteins.

EPR Spectroscopy of the Hexaaquaferrous Complex

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The Fe(II) ion ($3d^6$) is of intrinsic interest because of its importance in biochemistry, and in other areas, such as in the construction of paramagnetic polynuclear transition metal clusters. The free ion is not normally found, but rather Fe(II) is coordinated by various ligands in differing configurations. One of the most prevalent is the six-fold octahedral coordination, of which the hexaaquaferrous complex $[\text{Fe}(\text{H}_2\text{O})_6]^{2+}$ is a prime example. Despite its importance, the magnetic properties of the hexaaquaferrous complex have not been well characterized until recently. Only in the past few years, EPR detection of that ion in its high-spin ($S = 2$) form has become a possibility [1-3] because of the technological progress of high-frequency EPR techniques. Still, EPR detection of the hexaaquaferrous complex has been fraught with difficulties, as witnessed by the quoted references. In the present work we investigate this complex as a salt with the hexafluorosilicate counterion, $[\text{Fe}(\text{H}_2\text{O})_6]\text{SiF}_6$. It turns out that this particular salt exhibits optimal properties for detecting and characterizing the $[\text{Fe}(\text{H}_2\text{O})_6]^{2+}$ ion by means of electron magnetic resonance, both in zero-, and high-field. Very interestingly, the spin Hamiltonian parameters characterizing the ground $S = 2$ state of the complex vary strongly depending whether a pure complex is investigated, or it is doped into a diamagnetic analog, $[\text{Zn}(\text{H}_2\text{O})_6]\text{SiF}_6$. Thus, for the pure complex, the zero-field splitting parameters are: $D = +12.0 \text{ cm}^{-1}$, $E = 0.65 \text{ cm}^{-1}$ while for $[\text{Fe}(\text{H}_2\text{O})_6]^{2+}$ doped into $[\text{Zn}(\text{H}_2\text{O})_6]\text{SiF}_6$ at 8% concentration the same parameters are: $D = +13.4 \text{ cm}^{-1}$, $E = 0.13 \text{ cm}^{-1}$, indicating a significant decrease of the zfs tensor rhombicity in the latter case. The meaning and significance of this observation will be discussed in the presentation.

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Dipolar Encoded ^1H - ^{15}N HETCOR Spectroscopy for Structural Characterization of Aligned Samples

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Polarization inversion spin exchange at the magic angle (PISEMA), [1] which correlates the orientational-dependent, anisotropic ^1H - ^{15}N heteronuclear dipolar couplings and ^{15}N chemical shifts, has been widely used for structural characterization of aligned samples, such as membrane proteins/polypeptides in 'native-like' lipid bilayers. PISEMA spectra from α -helices feature specific wheel patterns, which can be directly analyzed to obtain tilt, polarity, and high-resolution structures. So far, the orientational-dependent anisotropic ^1H chemical shifts, which provide complementary information for structural analyses including the hydrogen bonding geometry, have not been used for such structural characterization. Here, we use two-dimensional (2D) heteronuclear correlation (HETCOR) spectroscopy to correlate the anisotropic ^1H chemical shifts and ^{15}N chemical shifts of NH amide groups. We take advantage that when the ^{15}N decoupling is absent during the ^1H chemical shift evolution, the ^1H chemical shift resonances are split into two separated by the ^1H - ^{15}N heteronuclear dipolar couplings. Thus, the dipolar encoded HETCOR spectra allow ones to obtain not only the ^1H - ^{15}N heteronuclear dipolar couplings and the ^{15}N chemical shifts as in PISEMA spectra, but also the ^1H chemical shift restraints from peptide planes [2].

In this presentation, we will demonstrate the advantages of this HETCOR method and its applications to the structural characterization of peptides/proteins aligned in lipid bilayers.

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Protein NMR under Physiological Conditions

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My laboratory is using high-resolution NMR to explore protein structure, stability, and dynamics in living cells and under crowded conditions *in vitro*. We became interested in this area after reading the work of Allen Minton. Allen was one of the first to realize that protein chemistry should be affected by the crowded conditions inside cells, where proteins comprise 30% of a cell's volume and reach concentration in excess of 300 g/L. For comparison, the protein concentration of egg white is 150 g/L.

One challenge of our work is resolving signals of the protein being studied from signals of the other molecules in the cell. I will discuss the techniques we use, our work on intrinsically disordered proteins in *Escherichia coli*, our effort to move these protein NMR experiments into animal cells, and our work on globular protein stability and dynamics under crowded conditions *in vitro*.

The Origins of Iron and NO for Biological Dinitrosyliron Complexes

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The EPR-visible “ $g=2.03$ signal” has been detected in a multitude of biological systems, and has been correlated with a diverse array of pathological conditions involving nitric oxide (NO), including cancer, inflammation, autoimmune disease, neurodegenerative illness, and cardiovascular disease. Its possible biological function(s) is unknown, due in large part to the lack of information on its chemical identity. We report the results of studies using isolated murine cells that demonstrate (1) the iron for these complexes originates from the “chelatable iron pool”, which is the central player in the control of cellular iron homeostasis; (2) the nitrosyl ligands originate from only free NO, not from other possible nitrogen oxide species, and (3) the entirety of this cellular iron pool is converted to these paramagnetic species. These results have important implications for the roles of NO in cellular injury from reactive oxygen and nitrogen species, iron homeostasis, and the utility of this signal as a marker for free NO exposure.

Ferromagnetic resonance force microscopy

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Magnetic resonance force microscopy (MRFM) is a new three-dimensional imaging technique probing the dynamic magnetic properties of samples. The extremely high sensitivity combined with the high spatial resolution of this technique makes it a promising candidate for the characterization of spintronic devices. So far research involving magnetic resonance force microscopy has focused on improving force sensitivity and characterization of paramagnetic samples, for which single spin sensitivity has been achieved recently. Some of the challenges of magnetic resonance force microscopy investigations of spintronic devices will be discussed, including the separation of local and global information contained in a typical MRFM spectrum of a ferromagnetic sample. The local information can be utilized to obtain spatially resolved information about the dynamic magnetic properties of the sample.

A Low-E Probe for ^{19}F - ^1H NMR in Dilute Biological Solids

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Sample heating induced by radio frequency (RF) irradiation presents a significant challenge to solid state NMR experiments in proteins and other biological systems, causing the sample to dehydrate and resulting in distorted spectra and a damaged sample. We will describe a large volume, low-E ^{19}F - ^1H solid state NMR probe, which we developed for static 2D ^{19}F CPMG studies of dilute membrane proteins in an electrically lossy environment at 600 MHz field. In ^{19}F -CPMG and related multi-pulse ^{19}F - ^1H experiments the sample is heated by the conservative electric fields E produced in the sample coil at both ^{19}F and ^1H frequencies. Instead of using a traditional single coil approach, our low-E ^{19}F - ^1H probe utilizes two orthogonal loop-gap resonators in order to minimize the conservative electric fields responsible for sample heating. Absence of the wavelength effects in loop-gap resonators results in very homogeneous RF fields and enables the study of large sample volumes, an important feature for the dilute protein preparations. The orthogonal resonators provide intrinsic isolation between the ^{19}F and ^1H channels, which is another major challenge for the ^{19}F - ^1H circuits where Larmor frequencies are only 6% apart. We also detail steps to reduce ^{19}F background signals through careful choice of materials and demonstrate application of the probe for 2D ^{19}F CPMG spectroscopy in oriented lipid membranes.

Multi-Frequency Pulsed EPR and ENDOR at 120, 240, and 336 GHz, and the prospects of high power pulsed EPR at frequencies up to 1200 GHz.

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Driven by possible applications of spin qubit quantum computing, single molecule magnets, and nanotechnology there is a renewed interest in the dynamics of spin systems at high fields and frequencies. Using solid state sources it is now possible to achieve pulsed magnetic resonance up to 340 GHz and possibly even higher frequencies. At the National High Magnetic Field Laboratory we have constructed a pulsed EPR instrument that operates at 120, 240, and 336 GHz. Initial results of pulsed EPR in a range of systems will be presented. The advantages of a high-field instrument for pulsed ENDOR will be illustrated by a pulsed ENDOR study at 240 GHz of the ^{39}K hyperfine and quadrupole interaction in the $\text{K}_3\text{NbO}_8:\text{Cr}^{5+}$ system.

While, in principle, high frequencies enable pulsed measurements with a high bandwidth and thus excellent time resolution, the low available powers at terahertz frequencies has hampered the development of high power pulsed very high frequency instruments. A possible avenue, which is currently actively pursued at the maglab, lies in the utilization of a Free Electron Laser which can deliver kW power pulses at frequencies up to 3 THz. In combination with the available fields of up to 45 Tesla, this could lead to pulsed EPR measurements at 1.2 THz with sub-nanosecond time resolution. Not only can this be important for the study of protein dynamics of aqueous solution, but this can also have a high impact in the study of materials with relatively fast relaxation rates, like organic conductors or transition metal ion complexes.

NMR-based Marine Environmental Metabolomics at the Hollings Marine Laboratory

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Management of the nation's marine environmental resources poses inherently complex questions. Application of "-omics" technology to understanding the effects of environmental stressors on marine species may be a way to augment or improve the environmental decision making process. At the Hollings Marine Laboratory (HML), a multi-institutional research facility located in Charleston, SC, efforts in genomics, proteomics, metalomics and metabolomics are ongoing.

This presentation will give an overview of the application of NMR-based metabolomics to marine species from mammals to microbes. These experiments are being executed at the new HML NMR Facility at 700 and 800 MHz. By leveraging the expertise of the core of marine researchers at the HML, including biologists, toxicologists, ecologists and chemists, value is being added to these expert's research programs while providing the opportunity to move techniques and tools that have been developed primarily for human health research into challenging new areas.

Modern High - Field EPR and NMR , Their Complementary Nature and Applications to Single Molecule Magnetism

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Studies of materials with electronic spins as high as 35 and large zero-field splittings require specialized instrumentation, not normally available commercially. EPR measurements on such materials require microwave frequencies up to 500-1000 GHz and magnetic fields up to 30 tesla and higher. Also, many such materials have fast electronic spin-lattice relaxation such that the signals are observable only at liquid helium temperatures, 4 K or even lower. Similarly for their NMR studies, commercial instruments are unsuitable because the line orders of magnitude wider than can be measured with commercial spectrometers. In this talk we will discuss the EPR and NMR facilities that have been developed at the National High Magnetic Field Laboratory in Tallahassee , and which are quite suitable for such studies. In this talk we will provide some details of the locally-developed NMR and EPR instrumentation, and how each technique provides complementary information on various facets of a magnetic compound. Specific examples will be discussed from the class of materials known as single molecule magnets (SMMs).

Biophysical Characterization of the GM2 Activator Protein with Phosphatidylcholine Bilayers

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The GM2 Activator Protein (GM2AP) is an accessory protein involved in the catabolism of ganglioside GM2. A goal of our research is to determine the membrane bound orientation of GM2AP on phosphatidylcholine bilayer surfaces. We currently utilize both site-directed spin labeling electron paramagnetic resonance spectroscopy and intrinsic tryptophan fluorescence to accomplish this goal. Results demonstrating feasibility of site-directed spin labeling approach, proper protein folding and surface association are presented. Specifically, power saturation experiments utilizing paramagnetic colliders of different solubilities in the aqueous and hydrocarbon phases indicate that two cysteine mutants of GM2AP, A60R1 and N136R1, which have been suggested to be located in putative membrane binding loops, are localized at the surface of model membranes, but do not penetrate into the bilayer. Additionally, GM2AP contains three tryptophan residues, with two located in the putative membrane binding loops. Intrinsic tryptophan fluorescence quenching, intensities and wavelength shifts upon binding to POPC bilayers confirm a surface association for GM2AP.

Recent Developments in High-Frequency EPR at the University of Florida

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I will describe a number of fairly unique high-frequency EPR capabilities that have been developed at the University of Florida during the course of the past several years, many of which can also be used in conjunction with the high-field resistive magnets at the National High Magnetic Field Laboratory (NHMFL) in Tallahassee. The use of a vector network analyzer (with superheterodyne receiver), together with various sweepable solid-state sources and multipliers, enables frequency coverage spanning the entire range from 8 to 715 GHz (these are the extremes for which we have obtained high-quality EPR spectra). At the lower frequency end of this range, we make use of oversized cylindrical resonators (coupled to the spectrometer via rigid rectangular waveguide), enabling studies of extremely small single crystal samples (< 0.1 mg). Coupling through the side-walls of a horizontal cylindrical resonator enables in-situ rotation of one of the end-plates which, when combined with a split-pair magnet, allows for double-axis rotation of highly anisotropic samples relative to the applied magnetic field. We have also recently re-designed one of these cavities to hold a cylindrical plastic diamond-anvil pressure cell. While many components in the cell give rise to EPR signals close to $g = 2$, the cell itself is not so lossy at liquid helium temperatures. Therefore, we have been able to study the effects of pressure (so far up to 6 kbar) on the EPR spectrum of a molecular nanomagnet with significant zero-field splitting. All of these cavity-based techniques work up to about 350 GHz, are compatible with the DC magnets at the NHMFL (including the 45 T hybrid), and can be cooled to temperatures as low as 500 mK.

At higher frequencies, we make use of low-loss corrugated HE_{11} mode waveguide to couple the spectrometer to the high-field environment via a quasi-optical bridge employing corrugated horns, a wire-grid polarizing beam splitter, and focusing mirrors. A replica of the system used in Gainesville is also stationed permanently at the NHMFL DC field facility in Tallahassee. Using this approach, we have obtained high-quality EPR spectra on mg sized single-crystal samples up to a frequency of 715 GHz (limited mainly by the bandwidth of the corrugated tube). Very recently we have combined this approach with other techniques, enabling pump-probe-type experiments, where the microwaves incident on the sample at the end of the HE_{11} tube may play both the role of the pump and the probe. In the former case, we use micro-Hall sensors to probe changes in magnetization; in the latter case, we use surface acoustic waves to trigger rapid magnetization reversal in crystals of single-molecule magnets, and then use high-frequency EPR (250-350 GHz) to monitor the system as it equilibrates. Time permitting, I will show as many examples as possible using the above techniques.

EPR Studies of Photosystem II and Artificial Systems

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Photosystem II (PSII) uses light energy to split water into protons, electrons and O₂ (1). In this reaction, Nature has solved the difficult chemical problem of efficient four-electron oxidation of water to yield O₂ without significant amounts of reactive intermediate species such as superoxide, hydrogen peroxide and hydroxyl radicals. In order to use Nature's solution for the design of artificial catalysts that split water, it is important to understand the mechanism of the reaction. One important question is how the substrate waters are coordinated to the Mn₄Ca cluster in the O₂-evolving complex (OEC) of PSII. Recently, we have used electrospray ionization mass spectrometry to determine the rates of μ-oxo exchange in high-valent oxo-bridged multinuclear manganese model complexes (2-3). The observed rates provide strong evidence that the substrate waters are bound to the metal ions in the OEC as terminal ligands. These studies have also yielded a new method for incorporation of isotopically enriched oxygen into the di-μ-oxo bridges of Mn^{III}-Mn^{IV} model complexes. Using this method to prepare ¹⁷O-labeled samples and ENDOR spectroscopy, we have measured the ¹⁷O hyperfine coupling of the bridging oxos to manganese in a Mn^{III}-Mn^{IV} model complex. These results are significant for determination of the core electronic structure of the OEC. Supported by the National Institutes of Health grants GM32715 (G.W.B.) and EB00326929 (C.P.S.).

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Imaging Electron Transfer Pathways in Natural Photosynthesis Using Time-Resolved High-Field EPR/ENDOR Spectroscopy

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In photosynthesis, the primary energy conversion reactions involve a photoinitiated sequence of efficient electron-transfer (ET) steps that result in charge separation across a biological membrane, thus converting light into an electrochemical potential. These biological ET reactions occur between cofactors that are encased within well-ordered reaction center (RC) protein “scaffolds”. Up until now we were limited in both the ability to differentiate between ET pathways thru the RC and to determine the role of local protein environment in fine tuning the high efficiency of solar energy conversion.

Using time-resolved High-Frequency (HF) EPR spectroscopy, we have developed new approaches which enable us to detect magnetic interactions between electron donor-acceptor cofactors and amino acid environments, thus allowing ET pathways to be mapped and the geometry of the charge-separated states to be reconstructed. In the first example, isotopic substitution in combination with the time-resolved HF ENDOR technique that allows us to localize the electron transfer pathways between primary and secondary acceptors in bacterial photosynthetic RC will be discussed. In the second example we will demonstrate how time-resolved HF EPR of spin-correlated radical pairs in photosystem I gives direct evidence that ET is bidirectional, proceeding along both cofactor branches A and B. These studies provide the groundwork for further development of the HF EPR techniques for elucidating details of the primary photosynthetic ET processes.

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Correlating the structure & function of oxidoreductases using REFINE'd EPR spectroscopy

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The system of mitochondrial enzymes and redox-carrier molecules, which ferry reducing equivalents from substrates to oxygen are collectively known as the electron transport system, or the respiratory chain. This system captures the free energy available from substrate oxidation so that it may later be applied to the synthesis of ATP. This chain can be dissected into four large multi-subunit complexes containing the principal respiratory carriers, named complex 1 to complex 4. The recent determination of the three-dimensional structure of many of these complexes has opened up the possibility to investigate the relationship between their structure and their catalytic function. This is an important issue as the mechanism of electron-coupled proton transfer in such membrane proteins is still poorly understood.

Quinones molecules are ubiquitous in living organisms and are found either within the lipid phase of the biological membrane (quinone pool) or are bound in specific binding sites within several of these membrane-bound protein complexes. The biological function of such bound quinones is determined by their ability to be reduced and/or oxidised in two successive one-electron steps. As a result, quinones are involved as one- or two-electron donors or acceptors in biological electron transfer. In this study, we focus on the redox-active ubiquinones from the cytochrome *bc*₁ complex (ubiquinol:cytochrome *c* oxidoreductase, QCR) from *S. cerevisiae* and in terminal oxidases from *E. coli*. The location and/or exact binding of such molecules are still unclear from several recent crystallographic studies.

Here we apply a range of EPR spectroscopic techniques to characterise these quinone-binding sites. High-field EPR spectroscopy is used to resolve the *g*-tensor, while pulsed Electron Nuclear Double Resonance (ENDOR) spectroscopy is used to map out the spin density distribution. Selective isotopes (¹³C, ¹⁵N, ²H, and ¹⁷O) and site-directed mutants are used to characterise binding within the protein by both ENDOR and 2-dimensional proton- HYperfine Sub-level CORrElation (HYSCORE) and DONUT spectroscopy. Further Electron Spin Echo Envelope Modulation (ESEEM) spectroscopy together with an inversion recovery filter (REFINE) is applied to resolve the question of whether the observed ¹⁴N modulations arise from interactions to semiquinones or to other underlying paramagnetic centres.

These results are compared with DFT calculations and corresponding information available on other protein binding sites and/or on model systems and are discussed with regard to the location and catalytic function of these quinones within their enzymes.

Photosynthetic Water Splitting: Investigations by Multifrequency Pulsed EPR Methods

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The Photosystem II component of oxygenic photosynthesis uses the full spectrum of visible light to split water with high quantum yield, providing activated protons and electrons, and producing molecular oxygen as a byproduct (albeit a crucial one). We are yet unable to develop synthetic systems that can carry out this important photochemical reaction with the yield and low energy photon range of this highly evolved natural system, and thus it is important for us to investigate its catalytic mechanism in detail. We are using a multifrequency pulsed EPR approach to this end, with a set of homebuilt and commercial instruments covering a frequency range from 8 to 130 GHz. These studies focus on the paramagnetic electron transfer intermediates in the water splitting reaction, including tyrosine radicals and a multinuclear manganese cluster. In addition to Photosystem II, we are targeting a variety of other metal and radical based enzyme systems, and I will provide some details on one or more of these systems as well.